SUPPLEMENT:

The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions, and increasingly important and numerous roles in health and disease

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A Primer on miRNA Biogenesis

Some miRNAs are distinct transcription units. Others are clustered and processed as a single polycistronic transcript.[1, 2] Approximately half of the known miRNAs are located in the introns of protein-coding genes[3-5] whereas the rest are intergenic.[6] MiRNAs are typically transcribed by RNA polymerase II (RPOL II), but exceptions have also been reported.[7, 8] This early double stranded RNA (dsRNA) transcript is named pri-miRNA, and is processed by the Drosha/DiGeorge-syndrome-critical-region-gene-8 (Drosha/DGCR8) complex in vertebrates or Drosha/Pasha in invertebrates[9] (Supp. Fig. 1a).

Drosha is an RNase III endonuclease that cleaves the pri-miRNA and produces a 75 nt pre-miRNA with a stem loop structure; DGCR8 is a double-stranded-RNA binding protein that guides Drosha. The pre-miRNA is exported from the nucleus to the cytoplasm by Ran-GTP and the transporter Exportin-5.[10-12] In the cytoplasm, the pre-miRNA is processed by DICER, which is another RNase III endonuclease.[13] DICER cleaves both ends of the pre-miRNA, leaving a dsRNA of ~19-23 nts in length with a 2-3 nt overhang on either side: this dsRNA product has traditionally been known as the miRNA:miRNA* duplex and is comprised of a driver (miRNA) and a passenger (miRNA*) strand. The driver strand is loaded onto the Argonaute protein (Ago), which is an essential component of the RNA-induced silencing complex (RISC) complex, whereas the passenger (miRNA*) is assumed to be degraded.[14] However, a recent study of miR* species showing a high degree of conservation among vertebrates and binding sites in the 3′ untranslated region (3′UTR) of mRNAs indicates that miR* could also contribute to regulation.[15] The miR/miR* is gradually being replaced by the more informative use of a -5p (resp. -3p) suffix to refer to the arm that actually gives rise to the mature miRNA product at hand.

In addition to this canonical (Drosha-, DGCR8- and DICER-dependent) pathway two more miRNA biogenesis pathways are known. The first of the two is Drosha-independent, DGCR8-independent, and DICER-dependent, and is represented by the ‘mirtrons’ (Supp. Fig. 1b).

Mirtrons are generated by the excision of introns of appropriate length from pre-mRNAs.[16] The second alternative pathway depends on Drosha and DGCR8 but is Dicer-independent and is exemplified by miR-451: in this case, Drosha generates shorter than usual pre-miRNAs that are loaded on Ago and sliced on their 3´ hairpin arm to yield 30-nt Ago-cleaved species; these are then subjected to 3´ resection activity that trims 7 nts leaving the predominantly cloned 23-nt product miR-451 (Supp. Fig. 1c). The nucleases required by this pathway remain to be identified.[9] There are also other minor pathways that generate miRNAs from tRNAs, snoRNAs, etc.[9]

Architecture of the *C13orf25* Transcript

The *C13orf25* gene is transcribed by RPOL II and codes for two transcripts: A and B. Transcript A (four exons - 965 nts) codes for a 32 amino acid (aa) protein whereas transcript B (two exons - 5058 nts) codes for a 70 aa protein. Interestingly, transcript B is a two-exon transcript that comprises precursors for 6 highly conserved miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1[17, 18] in its UTR. By virtue of its location, this group of miRNAs is *intergenic* and, borrowing from the names of its members, it has been referred to as the miR-17/92 cluster.

Genetic Dissection of the miR-17/92 Cluster

Early data showed over-expression of the miR-17/92 cluster in different tumors, namely, B-cell-lymphomas and lung and colon cancers.[19-21]

In one particular study of 46 lymphoma samples, which included 13 diffuse large B-cell lymphomas and 6 follicular lymphomas, 65% of the samples showed significant over-expression of miR-17/92. In another study, 15% of colorectal samples showed increased miR-17/92

expression.[19] These findings suggested that miR-17/92 might contribute to tumor development in hematopoietic and solid tumors.

The first experiments to implicate the miR-17/92 cluster in tumor progression employed a mouse model of B-cell lymphoma. Transgenic animals overexpressing the c-Myc oncogene driven by the immunoglobulin heavy-chain enhancer (Eμ) were used. These mice develop B-cell lymphoma by 4-6 months of age.[19] Hematopoietic stem cells (HSC) were obtained from fetal liver of transgenic animals and infected with a murine stem cell virus (MSCV) retrovirus that directs expression of the miR-17/19b-1 portion of the cluster. Transfected cells were transplanted into lethally irradiated recipients. The miR-17/19b-1 portion of the cluster was used since it is a vertebrate-specific portion of the miR-17/92 cluster.

The results revealed that mice with reconstituted Eμ-myc/+HSC with control vector develop lymphomas in 3-6 months. However, mice with Eμ-myc/+HSC carrying miR-17/19b-1 develop B-cell lymphomas with shorter latency in approximately 51 days. The full-length miR-17/92 cluster was also tested and showed results similar to those of the truncated version of the miR-17/92 cluster. In addition, for the first time, suppression of apoptosis was proposed as the possible mechanism of miR-17/92's contribution to tumorigenicity.

This early study led to several important conclusions: 1) the miR-17/92 cluster has oncogenic properties; 2) a portion of the cluster (miR-17 + miR-19b-1) also has oncogenic properties; and, 3) suppression of apoptosis is a possible mechanism for the contribution of miR-17/92 to tumorigenesis. However, as the original report stressed,[19] the individual role of each member of the cluster still needs to be elucidated.

Subsequent studies of the function of individual miRNAs were facilitated by the study of the role of the miR-17/92 cluster's two paralogues. These paralogues, miR-106a/363 and miR106b/25 arose through gene duplication and deletion events during ancient vertebrate evolution. For those studies two knockout mice were generated: miR-106b/25$^{\Delta/\Delta}$ and

miR106a/363$^{\Delta/\Delta}$. Both mouse models were viable and fertile and lacked obvious physical abnormalities. Generated heterozygous mice (with one knockout allele and another wild-type) miR-17/92$^{+/\Delta neo}$ were born at the expected ratio, were viable and fertile, but were smaller in size than their wild-type littermates. In contrast, miR-17/92–deficient mice died shortly after birth. MiR-17/92-deficient embryos were 60% of the weight of their wild-type littermates at embryonic day E18.5 and necroscopy showed severely hypoplastic lungs and ventricular septal defect in the heart.[22]

Interestingly, deletion in mice of either miR-106b/25 or miR-106a/363 does *not* lead to developmental abnormalities, and these mice appear fertile.[22] Actually, only one allele of miR-17/92 is needed together with either miR-106b/25$^{\Delta/\Delta}$ or miR-106a/363$^{\Delta/+}$ to produce viable and fertile mice.[22] Deletion of the miR-17/92 cluster together with the paralogue miR-106b/25 (homozygous double knockout), or deletion of the miR-17/92 cluster together with paralogues miR-106b/25 and miR-106a/363 (homozygous triple knockout) leads to a more severe phenotype than the deletion of the miR-17/92 cluster alone and results in mouse death at E15. Double knockout mice show edema and vascular congestion at E13.5 and E14.5, which in turn leads to severe cardiac abnormalities. These results suggest the asymmetric and partial functional redundancy of clusters.[22]

Considering that miR-106a/363 is expressed in selected tissues in low quantity, that miR-17/92 and miR-106/363 are expressed ubiquitously, and that the miR-17/92 and miR-106a/363 clusters differ in miR-18, miR-19a, and miR-19b-1 it was postulated that these three miRNAs play a central role in tumorigenesis. Subsequently efforts supported this idea by showing that miR-17/92 plays a role in lymphoproliferative diseases,[23] meduloblastoma, and neuroblastoma[24, 25] and that deletion of miR-17/92 affects normal B-cell development.[22]

To dissect the role of individual components of the cluster, the B-cell Eμ-Myc mouse model was used. Two lymphoma lines were produced from conditional miR-17/92 knockout mice.[26]

First, upon deletion of the whole cluster in culture, cell proliferation was dramatically reduced. In the rescue experiment, upon introduction of the miR-17/92 cluster, the proliferation was restored. In the presence of the miR-17/92 cluster, the rate of apoptosis was significantly lower.[26] Members of the miR-17/92 cluster were shown to target the 'phosphatase and tensin homolog' PTEN,[26] a phospholipid phosphatase and a tumor suppressor.

PTEN negatively regulates the Phosphoinositide 3-kinase (PI3K) pathway, downstream targets of which are the Protein Kinase B (PKB), Akt and the mammalian target of rapamycin (mTOR). PTEN has been shown to be a miR-19b-1 target with two binding sites in its 3′UTR.[27-29] Consequently, miR-19b can activate the Akt-mTOR pathway both *in vitro* and *in vivo*. MiR-19b overexpression leads to increased phosphorylation of AKT in NIH-3T3 cells, as well as phosphorylation of S6 ribosomal protein.[26] *In vivo* experiments showed that *Eμ-myc-19b-1* and *Eμ-myc-17-19b-1* mice developed lymphomas whereas Western blotting and immunohistochemistry analyses showed high level of phosphor-S6 protein, the latter suggesting activation of the Akt-mTOR pathway through targeting miR-19b-1 of PTEN.[26] Other *in vivo* experiments showed that upon treatment with hydroxyurea Xenopus embryos exhibit signs of apoptosis.[26] MiR-19b-1 rescues the hydroxyurea treated embryo phenotype but mutated miR-19b-1 *is* unable to do the same.[26] In hydroxyurea-treated embryos injection with PTEN increases apoptosis; however, co-injection of PTEN with miR-19b-1 decreases apoptosis compared to injection with PTEN alone – mutant miR-19b-1 is again unable to show the same result as miR-19b.[26] These and other experiments show that miR-19b-1 is necessary and sufficient for recapitulating the oncogenic properties of the cluster and exerts its biological function through targeting of PTEN, which negatively regulates the PI3K-Akt-mTOR pathway.

Four series of mutant miR-17/92 cluster alleles were generated according to the four "seed" families - miR-17 (miR-17, miR-20a), miR-18 (miR-18), miR-19 (miR-19a, miR-19b-1) and miR-92 (miR-92) (Figure 3).[30] The wild-type and mutant alleles were cloned into a retroviral

vector, and the resulting construct was transduced into miR-17/92$^{\Delta/\Delta}$ lymphoma cells. Reintroduction of the full-length cluster conferred a growth advantage to miR-17/92$^{+/+}$ cells. Interestingly, the introduction of miRNAs *without* miR-19a and miR-19b-1 did not provide a growth advantage whereas reintroduction of *only* miR-19a and miR-19b-1 did.[30]

To determine whether miR-19a and 19b-1 are sufficient to recapitulate the phenotype of the whole cluster *in vivo,* Eμ-Myc nude mice were injected with miR-17/92$^{fl/fl}$ or miR-17/92$^{\Delta/\Delta}$.[30] Mice with miR-17/92$^{fl/fl}$ developed lymphoma within 2-3 weeks whereas miR-17/92$^{\Delta/\Delta}$ mice developed lymphoma with longer latency. Re-expression of the full-length cluster restored tumorigenesis as did introduction of miR-19a and miR-19b-1, but the latter did so less efficiently. Any combination that lacked miR-19 members failed to restore tumorigenicity.[30]

In summary, these studies show that miR-19a and miR-19b-1 are necessary and sufficient to recapitulate the oncogenic properties of the entire cluster.

The Members of the miR-17/92 Cluster Have Distinct Roles

The MiR-17/92 cluster has an established role as an "oncomir" and its dysregulation leads to overexpression, which promotes tumorigenesis. However, relatively recent studies showed exceptions to these rules: even though most of the miRNAs from the cluster exhibit a cooperative role in promoting cell survival, cell proliferation, and migration, each individual member of the cluster can play a different role in different cell types and different physiological contexts. Other evidence shows that overexpression of some miRNAs can lead to decreased cell proliferation and that down-regulation of some miRNAs leads to increased cell proliferation, cell survival and motility.

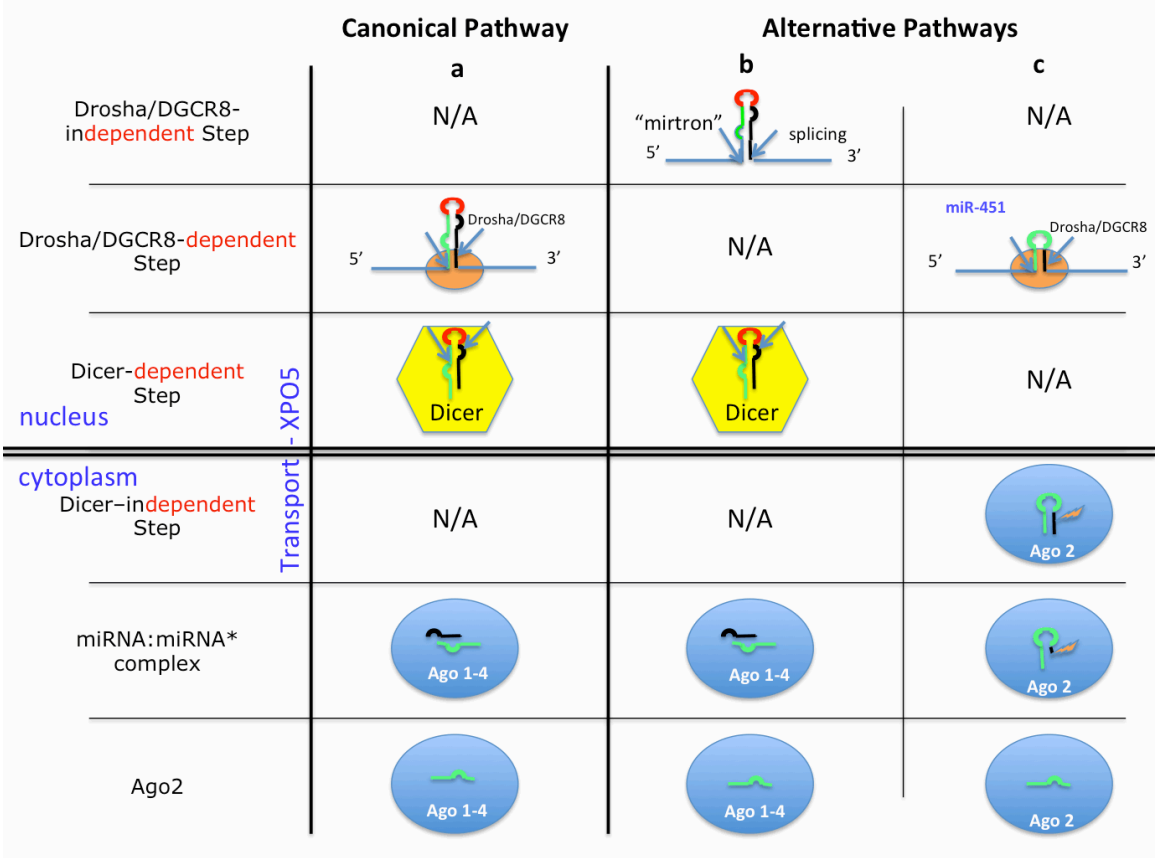
Mouse studies have shown that miR-17 overexpression *in vivo* leads to decreased proliferation, adhesion and migration. The same result was shown in a parallel study: introduction of miR-17 into breast cancer cell lines significantly reduced proliferation.[18, 31] A possible

mechanism could be through the suppression of E2Fs by miR17/92 via an auto-regulatory loop: miR-17 targets the amplified-in-breast-cancer (AIB1) gene, which is a transcriptional co-activator of E2F1.[18, 32, 33] Notably, 16.5 % of ovarian cancers, 21.9% of breast cancers, and 20% of melanomas show loss of the miR-17/92 cluster.[18, 34]

The Cluster as an Oncogene – More Details

Other hematopoietic tumors. In patients with multiple myeloma, it was shown that overexpression of miR-17, miR-20a, and miR-92-1 correlates with shorter progression free survival and poor prognosis.[35] A 5- to 17-fold increase in the expression of miR-19b-1 was detected in T-cell leukemia, namely in T-cell acute lymphoblastic leukemia (ALL).[36]

Other solid tumors. MiR-17 is significantly up-regulated in gastric cancer as revealed by microarray studies.[37] Also, miR-18a is up-regulated in nasopharyngeal carcinoma (NPC) samples.[38] In fact, higher levels of miR-18a are correlated with NPC advanced stage, lymph node metastasis, Epstein-Barr virus infection and a higher death rate from NPC, suggesting a role for the cluster in NPC development. Moreover, *in vitro* and *in vivo* assays demonstrated that miR-18a promoted the growth, migration and invasion of NPC cells by regulating the expression of DICER.[38] Transgenic mouse models of urothelial cancers were also found to display significant over-expression of miR-18a.[39] In prostate cancer, miR-106b/25 was shown computationally to target PTEN.[40] Lastly, the role of the miR-17/92 cluster in solid tumors was examined in multiple mouse models and shown to be highly context-specific.[25, 41]



Supp. Figure 1. Canonical and two alternative pathways of animal microRNA biogenesis. (**a**) Canonical pathway: it requires Drosha/DGCR8 cleavage in the nucleus followed by loop cleavage by Dicer in the cytoplasm and leads to the subsequent formation of a miRNA/miRNA* duplex with 2-3 nucleotides (nts) overhang; the driver strand of the duplex is loaded on the Argonaute protein (Ago), which is part of the RNA-induced silencing complex (RISC), whereas the passenger strand is assumed to be discarded. (**b**) "Mirtrons"- (**miR**-NA transcribed from in-**trons**) do not require Drosha/DGCR8 and are instead generated by excision of introns; mirtrons are Dicer dependent. (**c**) MiR-451 is an example of a second major alternative pathway of animal miRNA biogenesis. This pathway does not require Dicer. Drosha/DGCR8 cleaves a shorter than usual pre-miRNA, which is immediately loaded on Ago 2. Unidentified nucleases trim from the 3′ of this pre-miRNA until the pre-miRNA becomes 30 nts followed by further resection to a final mature miRNA that is 23 nts long.

List of Abbreviations for Supplement

3´UTR - 3´ untranslated region

aa – amino acids

Ago - Argonaute protein

AIB1- amplified-in-breast-cancer

ALL - Acute lymphoblastic leukemia

C13orf25 - chromosome 13 open reading frame 25

dsRNA - double stranded RNA

Drosha/DGCR8 - Drosha/DiGeorge-syndrome-critical-region-gene-8

HSC - hematopoietic stem cells

MIR17HG – miR-17/92 cluster host gene (non-protein coding)

miRNA - driver microRNA in miRNA:miRNA* duplex

miRNA* - passenger strand microRNA

miRNA:miRNA* duplex - double stranded RNA of ~19-23 nucleotides in length with a 2-3 nucleotides overhang on either side

mTOR - mammalian target of rapamycin

NPC - nasopharyngeal carcinoma

nts – nucleotides

PI3K - Phosphoinositide 3-kinase

PKB - Protein Kinase B, Akt

RPOL II - RNA polymerase II

PTEN - phosphatase and tensin homolog

RISC - RNA-induced silencing complex

UTR - untranslated region

Supplemental Material

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